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Specific Immunization Strategies Against Oxidized Low-Density Lipoprotein: A Novel Way to Reduce Nonalcoholic Steatohepatitis in Mice

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Nonalcoholic steatohepatitis (NASH) is characterized by hepatic lipid accumulation combined with inflammation, which can ultimately progress into cirrhosis. Recently, we demonstrated that deletion of scavenger receptors (SRs) CD36 and SR-A in hematopoietic cells reduced hepatic inflammation. In addition to uptake of modified lipoproteins, CD36 and SR-A are also involved in other functions that can activate the inflammatory response. Therefore, the actual trigger for SR activation during NASH is unclear. Here, we hypothesized that hepatic inflammation is triggered by recognition of oxidized LDL (oxLDL) by Kupffer cells (KCs). To inhibit recognition of oxLDL by KCs, low-density lipoprotein receptor (*Ldlr*$^{-/-}$) mice were immunized with heat-inactivated pneumococci, which were shown to induce the production of anti-oxLDL immunoglobulin M (IgM) antibodies, due to molecular mimicry with oxLDL. The mice received a high-fat, high-cholesterol diet during the last 3 weeks to induce NASH. Immunization with pneumococci increased anti-oxLDL IgM levels and led to a reduction in hepatic inflammation, as shown by reduced macrophage, neutrophil, and T cell infiltration, and reduced gene expression of tumor necrosis factor (*Tnf*), interleukin-6 (*Il-6*), interleukin-1β (*Il-1b*), monocyte chemoattractant protein 1 (*Mcp1*), and fibrosis-related genes. In immunized mice, KCs were smaller and showed fewer cholesterol crystals compared with nonimmunized mice. *Conclusion:* Antibodies to oxLDL play an important role in the pathogenesis of NASH. Therefore, the potential of phosphorylcholine-based vaccination strategies as a novel tool for the prevention and therapy of NASH should be tested in the future. (HEPATOLOGY 2012;56:894-903)

Nonalcoholic fatty liver disease (NAFLD) is a condition ranging from benign lipid accumulation in the liver (steatosis) to steatosis combined with inflammation. The latter is referred to as nonalcoholic steatohepatitis (NASH). NASH is considered as the hepatic component of the metabolic syndrome. Estimates from the United States are that 5.7%-17% of all adults have NASH, while 17%-33% of Americans suffer from NAFLD.[1,2] As obesity and insulin resistance reach epidemic proportions in industrialized countries, the prevalence of both NAFLD and NASH is increasing. NAFLD is therefore a major health hazard.[3] Steatosis alone is considered a relatively benign and reversible condition. However, the transition toward NASH represents a key step in the pathogenesis, as it sets the stage for the development of fibrosis, cirrhosis, and liver cancer. Although the mechanisms leading to steatosis are well described, little is known about the actual risk factors that drive hepatic inflammation during the progression toward NASH. Consequently, therapeutic options are limited. Therefore, knowledge about the events that lead to hepatic inflammation is of great importance for the diagnosis and treatment of NASH.

Recently, we demonstrated that deletion of scavenger receptors (SRs) CD36 and SR-A in

Abbreviations: apo, apolipoprotein; Col1A1, *collagen type 1A1; FFA, free fatty acid; HFC, high-fat, high-cholesterol; IgG, immunoglobulin G; IgM, immunoglobulin M;* Il-1b, *interleukin-1β,* Il-6, *interleukin-6; KC, Kupffer cell; LDL, low-density lipoprotein;* Ldlr$^{-/-}$, *low-density lipoprotein receptor;* Mcp1, *monocyte chemoattractant protein 1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; oxLDL, oxidized low-density lipoprotein; PC, phosphorylcholine; RLU, relative light unit; SR, scavenger receptors; TG, triglyceride;* Tnf, *tumor necrosis factor;* Tgf-β, *transforming growth factor β.*

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hematopoietic cells reduced hepatic inflammation.[4] In addition to uptake of modified lipids, scavenger receptors are involved in many other inflammatory pathways. These pathways include cellular adhesion, innate immune responses, and phagocytosis of apoptotic cells.[5] Based on the analogy between mechanisms for atherosclerosis and NASH, it is likely that the recognition of oxidized low-density lipoprotein (oxLDL) by Kupffer cells (KCs), rather than other pathways, is the actual trigger for scavenger receptor-mediated inflammation. Therefore, we hypothesized that hepatic inflammation is triggered by the recognition of oxLDL by KCs.

It has recently been shown that the levels of immunoglobulin M (IgM) autoantibodies to modified LDL are inversely correlated with atherosclerosis.[6-8] Oxidation-specific epitopes present in oxLDL are major targets of natural IgM antibodies.[9] These antibodies arise spontaneously without prior infection or immune exposure and mainly consist of the IgM isotype.[10] They are produced by innate-like B-1 cells and provide a first line of defense against bacterial and viral pathogens.[11,12] In addition, natural IgM antibodies play an important role in providing housekeeping functions by protecting from the accumulation of biological waste, such as oxLDL.[10] Upon oxidation of LDL, reactive oxidation products from phospholipids retain the intact phosphorylcholine (PC) headgroup, which becomes available for immune recognition. These PC headgroups represent one of many so-called oxidation-specific epitopes and are found on the outer side of the membrane of oxLDL.[13] A panel of monoclonal autoantibodies directed to epitopes of oxLDL was cloned from the spleens of apolipoprotein E ($apoE^{-/-}$) mice.[14] In particular, one immunodominant clonotypic set of IgM autoantibodies was identified, EO6, which was shown to specifically bind to the PC moiety of oxidized PC-containing phospholipids, such as those present in oxLDL.[13] EO6 antibodies were found to be identical to the natural T15 antibodies, which are germline-encoded natural antibodies exclusively derived from B-1 cells. These T15 antibodies protect mice against *Streptococcus pneumonia* infections, because PC is also present in the capsular polysaccharide of the cell wall of this bacterium. Based on this molecular mimicry, immunization of low-density lipoprotein receptor ($Ldlr^{-/-}$) mice with heat-killed *S. pneumonia* resulted in higher serum titers of anti-oxLDL IgM antibodies and decreased atherosclerosis.[15] These findings suggest that anti-oxLDL antibodies directed to the PC group present on oxLDL possibly inhibit the recognition of oxLDL by macrophage SRs such as CD36.

The aim of the current study was to determine whether oxLDL is causally involved in the pathogenesis of NASH. For this purpose, $Ldlr^{-/-}$ mice were used as a well-recognized model mimicking the human lipoprotein metabolism with high fidelity and is therefore also extremely useful to investigate the physiological triggers for hepatic inflammation, which can already develop upon short-term treatment with a high-fat, high-cholesterol (HFC) diet.[16] These mice were immunized with heat-inactivated pneumococci to investigate whether anti-oxLDL antibodies have a protective effect on NASH. Supporting our hypothesis, immunized $Ldlr^{-/-}$ mice showed reduced hepatic inflammation compared to non-immunized mice. These data demonstrate the importance of antibodies to oxLDL in the pathogenesis of NASH. Therefore, the potential of PC-based vaccination strategies as novel tool for the prevention and therapy of NASH should be tested in the future.

Materials and Methods

Preparation Immunogen. For immunization, the heat-inactivated R36A strain of *S. pneumoniae* (Birmingham, AL) was used, still bearing the PC headgroup epitope similar to oxLDL. Colonies of the R36A strain were harvested at mid-log phase after incubation at 37°C on blood agar plates and transferred to Todd-Hewitt plus 0.5% yeast broth. The mid-log phase is characterized by an opitcal density (OD) value of 0.425 to 0.45 at 600 nm. *S. pneumoniae* was heat-inactivated at 60°C for 30 minutes; afterward, no colonies of this suspension were detected on blood agar plates. For freezer stocks of strain R36A, small aliquots of *S. pneumoniae* at mid-log

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density were harvested and suspended in Todd-Hewitt plus 80% sterile glycerol and stored at −80°C.[17]

Mice, Immunization, and Diet. *Ldlr*$^{-/-}$ mice on a C57BL/6 background were housed under standard conditions and had access to food and water ad libitum. Experiments were performed according to Dutch laws and were approved by the Animal Experiment Committee of Maastricht University.

The immunization protocol started in 12-week-old female mice fed a normal chow diet. Mice were divided into four groups (n = 10 for each group) and received the equivilant of 10^8 colony-forming units of the heat-killed pneumococcal immunogen emulsified in 200 μL sterile 0.9% NaCl for the primary subcutaneous immunization, subsequently three intraperitoneal booster immunizations were administered every 2 weeks.[15] The control group received an NaCl injection only. After immunization, the mice were given normal chow, the control group, or a HFC diet, the experimental group, for 3 weeks. Blood from the tail vein was collected after the dietary period and mice were then sacrificed by cervical dislocation. Liver tissue was isolated and snap-frozen in liquid nitrogen and stored at −80°C or fixed in 4% formaldehyde/PBS. The collection of blood and specimens, the biochemical determination of lipids in plasma and the liver, liver histology, alanine aminotransferase, RNA isolation, complementary DNA synthesis and quantitative polymerase chain reaction and auto-antibody titers against IgG and IgM antibodies to CuOx-LDL and malondialdehyde-LDL have been described extensively.[4]

Immune complex measurements were performed as described.[15] Briefly, circulating immune complexes were determined by a capture assay in which a polyclonal antibody specific for murine apoB100 was coated on microtiter wells at 5 μg/mL in phosphate-buffered saline. Individual mouse sera (1:100) were added to the wells and incubated for 1 hour at room temperature. IgM bound to the captured apoB-containing particles was detected using an alkaline phosphatase–conjugated goat anti-mouse IgM antibody by chemiluminescent enzyme-linked immunosorbent assay. The amount of IgM bound to the captured LDL was then normalized for the amount of captured apoB and expressed as a ratio of IgM counts (relative light units/100 ms) to apoB100 counts (relative light units/100 ms) or IgM/apoB.

Electron Microscopy. A detailed overview about the postfixation, embedding, cutting, and type of electron microscope has been described.[16] To stain the KC lysosomes, acid phosphatase enzyme cytochemistry was performed. Small wedge biopsies of the liver were perfused by syringe injection with ice-cold 2% purified glutaraldehyde in 0.1 M cacodylater buffer (pH 7.4) for 15 minutes. The wedge biopsies were cut into small pieces and kept in 0.1 M cacodylate buffer plus 7.5% sucrose at 4°C until further processing; the buffer solution was refreshed weekly. The samples were frozen for 1 hour at −30°C whereafter 50-μm-thick cryosections were made. These sections were incubated according to the cerium-based method of Robinson and Karnovsky for the localization of acid phosphatase.[32] After incubation, the sections were washed two times in 0.1 M cacodylate buffer supplemented with 5% sucrose, refixed in 3% glutaraldehyde in cacodylate buffer for 1 hour and rinsed overnight in veronal acetate buffer (pH 7.4, 4°C). The sections were then postfixed for 30 minutes in 2% osmium tetroxide in veronal buffer plus 4% sucrose and then routinely processed for embedding in epon.

Statistical Analysis. Data were statistically analyzed by performing two-tailed nonpaired *t* tests using GraphPad Prism version 4.03 for Windows. Data are expressed as the mean ± SEM and were considered significant at $P < 0.05$.

Results

Increased IgM Antibody Titers Against Modified LDL After Immunization with Heat-Inactivated Pneumococci. To determine whether IgM autoantibodies to oxLDL have a protective effect on liver inflammation, mice were immunized for 9 weeks with heat-inactivated pneumococci, known to induce high anti-oxLDL IgM titers dominated by T15-idiotypic IgM. To induce NASH, the mice received an HFC diet during the last 3 weeks. Total body weight and the ratio of liver weight to total body weight were not significantly different between the different groups (Supporting Fig. 1). Immunization of *Ldlr*$^{-/-}$ mice with heat-inactivated pneumococci resulted in a strong increase in IgM titers to oxLDL (Fig. 1A,B). Only weak but significant IgG responses were observed, consistent with previous reports that pneumococcal immunizations induce an IgM-dominated thymus-independent type-2 response highly specific for PC (Fig. 1C,D). The levels of circulating IgM/apoB immune complexes did not differ between the groups, likely indicating efficient clearance of oxLDL (Supporting Fig. 2).

No Difference in Liver Lipid Levels Between Immunized and Nonimmunized Ldlr$^{-/-}$ Mice After 3 Weeks of HFC Diet. To investigate liver lipid levels in hyperlipidemic mice with or without immunization, biochemical assessment of liver cholesterol, triglycerides

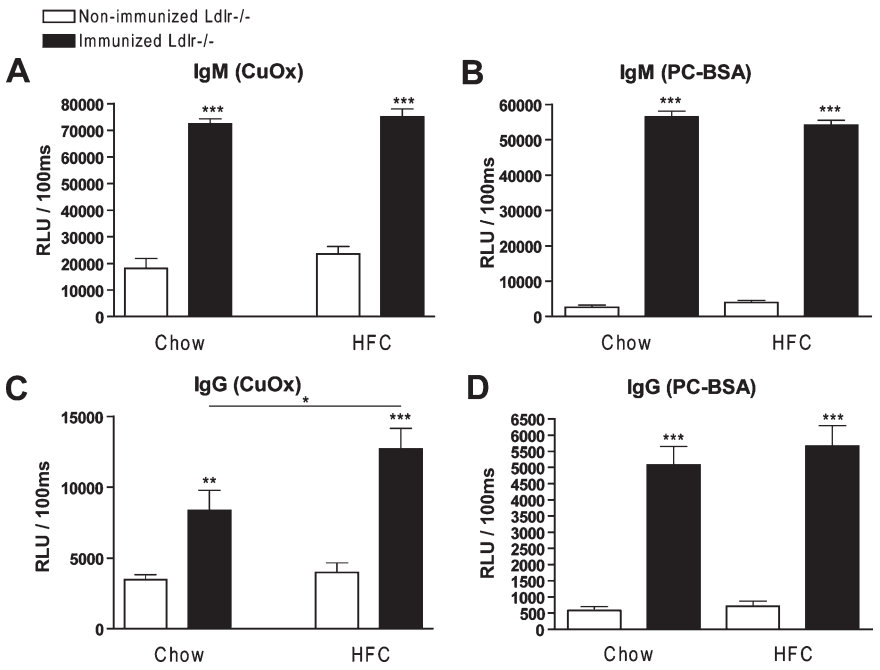

Fig. 1. IgM autoantibodies in mice that received pneumococcal immunization. (A-D) IgM and IgG antibodies against oxLDL (CuOx and PC-BSA) were measured in plasma of pneumococci-immunized (n = 10) and control (n = 10) mice at a dilution of 1:200, respectively. Data are expressed as relative light units (RLU)/100 ms and were triplicate determinations. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

(TGs), and free fatty acids (FFAs) was performed (Fig. 2A). After 3 weeks on an HFC diet, a clear increase in all liver lipid levels was observed compared with mice on a chow diet. Liver lipid levels did not differ between immunized and nonimmunized $Ldlr^{-/-}$ mice on the HFC diet. Mice on the chow diet showed a small increase in liver lipid levels after immunization when compared with nonimmunized $Ldlr^{-/-}$ mice. Oil red O and hematoxylin and eosin staining confirmed the biochemical liver lipid measurements (Fig. 2B-E and Supporting Fig. 3).

Decreased Plasma Cholesterol in Immunized $Ldlr^{-/-}$ Mice on an HFC Diet Compared with Control Mice. The effect of immunization on plasma lipids was assessed by measuring the levels of plasma cholesterol, TGs, and FFAs. After feeding on the HFC diet, a significant increase was observed for all plasma lipids compared with mice on a chow diet. Interestingly, plasma cholesterol was reduced in immunized $Ldlr^{-/-}$ mice compared to nonimmunized mice on an HFC diet. Plasma TGs and FFAs did not differ between the groups following an HFC diet. On chow diet, plasma lipid levels did not differ between the groups (Fig. 3).

Decreased Hepatic Inflammation in $Ldlr^{-/-}$ Mice Immunized with Heat-Inactivated Pneumococci. To determine whether immunization of $Ldlr^{-/-}$ mice with heat-inactivated pneumococci affects hepatic inflammation, liver sections were stained for the inflammatory cell markers Mac-1 (infiltrated macrophages and neutrophils), NIMP (neutrophils), and CD3 (T cells). As shown in Fig. 4A, the number of infiltrated macrophages, neutrophils, and T cells was lower in immunized $Ldlr^{-/-}$ mice compared with nonimmunized mice after feeding on an HFC diet. These data on cell infiltration are confirmed by hematoxylin and eosin staining (Supporting Fig. 3). Moreover, the normal chow diet induced a significant increase in the number of neutrophils in immunized chow-fed mice compared with nonimmunized mice. Representative histological pictures of the Mac-1 staining for all four experimental groups are shown in Figure 4B-E. Further confirming the reduced hepatic inflammation in immunized $Ldlr^{-/-}$ mice on the HFC diet, gene expression analysis showed a significant decrease in the inflammatory markers tumor necrosis factor (*Tnf*), interleukin-1β (*Il-1b*), interleukin-6 (*Il-6*), and monocyte chemoattractant protein 1 (*Mcp1*) in livers of immunized $Ldlr^{-/-}$ mice on an HFC diet compared with nonimmunized mice (Fig. 4F). However, hepatic inflammation in $Ldlr^{-/-}$ mice on an HFC diet after immunization was still higher than chow-fed immunized mice according to the inflammatory markers *Tnf*, *Il-1b*, and *Mcp1*. The presence of elevated aminotransferases in plasma-like alanine aminotransferase did not differ between the different groups (Supporting Fig. 4).

After 3 Weeks of HFC Diet, Immunization Prevented Expression of Fibrosis-Related Genes in $Ldlr^{-/-}$ Mice. Fibrosis is considered an advanced stage of NASH. Collagen staining (sirius red) was performed

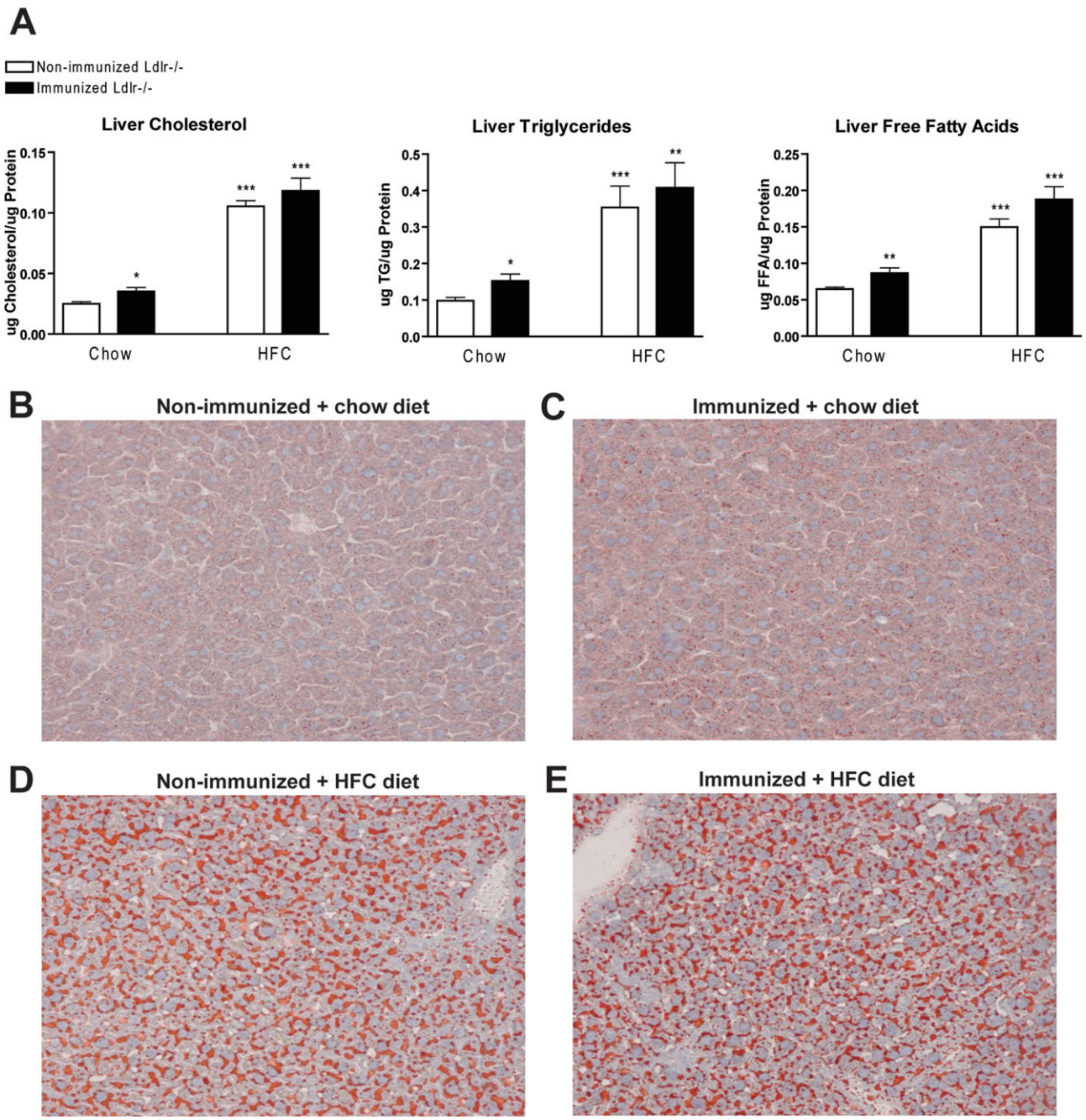

Fig. 2. Liver lipid levels. (A) Liver cholesterol, TGs, and FFAs after chow and 3 weeks of an HFC diet. (B-E) Oil red O staining after 3 weeks of an HFC diet in (B,D) nonimmunized (C,E) and immunized $Ldlr^{-/-}$ mice after (B,C) chow and (D,E) 3 weeks of feeding on an HFC diet, respectively. Asterisks indicate significant difference from nonimmunized mice on a chow diet. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

to determine the degree of fibrosis. No differences were observed between the experimental groups after 3 weeks of an HFC diet (Fig. 5A), which is probably related to the short duration of the HFC diet. However, gene expression analysis for collagen type 1A1 (*Col1A1*) and transforming growth factor β (*Tgf-β*) demonstrated that the messenger RNA levels of these fibrogenic genes were lower in immunized mice compared with nonimmunized mice on an HFC diet (Fig. 5B).

Decreased Foamy Appearance of KCs in Immunized $Ldlr^{-/-}$ Mice on an HFC Diet. Immunohistochemistry for CD68 was performed to characterize the KCs. Scoring of the CD68 positive sections revealed a reduction in size of foamy KCs in immunized $Ldlr^{-/-}$ mice compared with nonimmunized mice on an HFC diet (Fig. 6A-C). Gene expression of *Cd68* was reduced in the immunized $Ldlr^{-/-}$ mice compared with nonimmunized mice on an HFC diet (Fig. 6B).

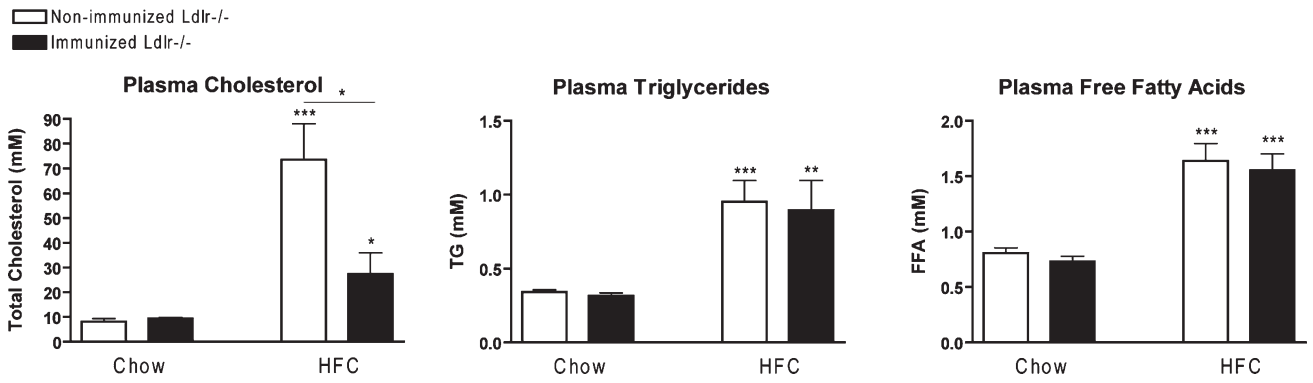

Fig. 3. Plasma lipid levels. Plasma cholesterol, TGs, and FFAs after a chow diet and 3 weeks of an HFC diet in nonimmunized and immunized $Ldlr^{-/-}$ mice. Asterisks indicate significant difference from nonimmunized mice on a chow diet. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Electron microscopy of KCs confirmed the differences in size of the KCs between immunized and nonimmunized $Ldlr^{-/-}$ mice, and showed that the immunized mice on an HFC diet had less lysosomal cholesterol accumulation and cholesterol crystals compared with nonimmunized mice (Fig. 6D).

Discussion

Until now, the actual risk factors that drive hepatic inflammation during the progression to NASH were unknown. To determine whether oxLDL is causally involved in the pathogenesis of NASH, serum anti-oxLDL IgM antibody levels were increased by immunizing $Ldlr^{-/-}$ mice with heat-inactivated pneumococci, which dramatically decreased hepatic inflammation. These data point toward oxLDL as a trigger for hepatic inflammation. Furthermore, our data suggest that PC-based vaccination strategies could be the basis for a vaccination protocol for NASH therapy. However, the long-term consequences of immunization are unknown at the moment and should be tested in the future.

SR-mediated uptake of oxidized lipoproteins by macrophages sets off a cascade of proinflammatory events leading to the initiation of the inflammatory response. oxLDL is phagocytosed by macrophages via binding of the oxPC molecules present in oxLDL to macrophage SRs and results in foam cells.[18,19] Furthermore, CD36 has been implicated in inflammatory signaling induced by oxLDL.[20] Previously, we have shown that both CD36 and SR-A play an important role in diet-induced NASH.[4] Because scavenger receptors have a wide spectrum of functions,[21-23] it is not clear whether the recognition of modified lipoproteins is the actual trigger for hepatic inflammation during NASH. Our data demonstrate for the first time that inflammation is reduced in the livers of pneumococci-immunized mice. These results are in line with earlier findings demonstrating decreased atherosclerotic lesion formation after pneumococcal immunization.[15] Similarly, $apoE^{-/-}$ mice immunized with PC, one of the epitopes of anti-oxLDL autoantibodies present in oxLDL but also in the CPS of *S. pneumoniae*, demonstrated an increase in anti-oxLDL autoantibodies together with a reduction in atherosclerotic lesions.[24] Several in vitro studies suggest that the induced IgM antibodies against oxLDL prevent binding and uptake of oxLDL by macrophages and/or neutralize its proinflammatory signaling.[11,15,25,26] Indeed, the inflammatory process associated with atherosclerotic plaque formation is linked to the cytotoxicity and macrophage chemo-attractivity of oxLDL. Moreover, oxLDL is thought to be an atherogenic factor, because its uptake by macrophages results in the formation of foam cells, the hallmark cells of atherosclerotic lesions.[18,19,27] Our data provide evidence for similar mechanisms between atherosclerosis and NASH. Thus, the reduced inflammation in mice in which the SRs on hematopoietic cells had been deleted is likely to be related to the reduced recognition of oxLDL by KCs.

Interestingly, plasma cholesterol levels were significantly reduced in our immunized $Ldlr^{-/-}$ mice. Previously, it was shown that anti-oxLDL antibodies directed to the PC group present on oxLDL inhibit the recognition of oxLDL by macrophage SRs.[28] We speculate that the formed immune complexes containing both LDL and oxLDL particles may be cleared faster by alternative pathways, as this is the case for IgM mediating apoptotic cell clearance. However, measurements of IgM/apoB immune complexes indicated that there were no differences between our groups of $Ldlr^{-/-}$ mice, also suggesting that the induced IgM mediate their protective effect by directly neutralizing the proinflammatory effects of oxLDL. These findings are in line with Binder et al.,[15] who

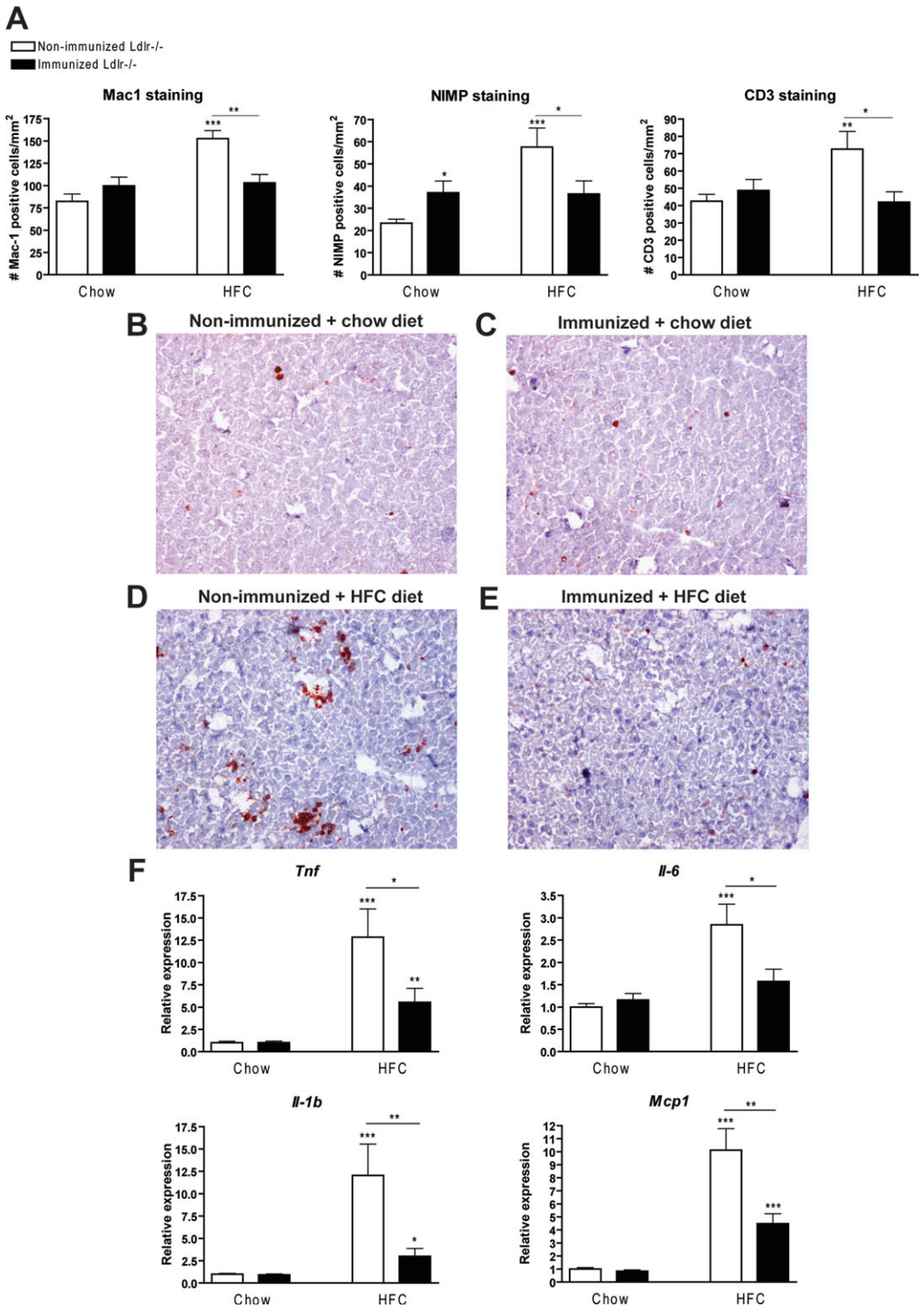

Fig. 4. Parameters of hepatic inflammation. (A) Liver sections were stained for infiltrated macrophages and neutrophils (Mac-1), neutrophils (NIMP), and T cells (CD3) and counted. (B-E) Representative images of Mac-1 staining (magnification ×200) after feeding on a chow (B,C) and an HFC diet (D,E) in nonimmunized (B,D) and immunized (C,E) $Ldlr^{-/-}$ mice, respectively. (F) Gene expression analysis for *Tnf*, *Il6*, *Il1β*, and *Mcp1*. Asterisks indicate significant difference from nonimmunized mice on a chow diet. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

demonstrated that under these conditions, no differences in immune complexes were observed. It is possible that the protective effect of these antibodies in vivo is further enhanced via a reduction in plasma cholesterol levels, since plasma cholesterol levels are an important trigger for hepatic inflammation.[16]

NASH patients are often associated with high levels of lipid peroxidation products such as those present in

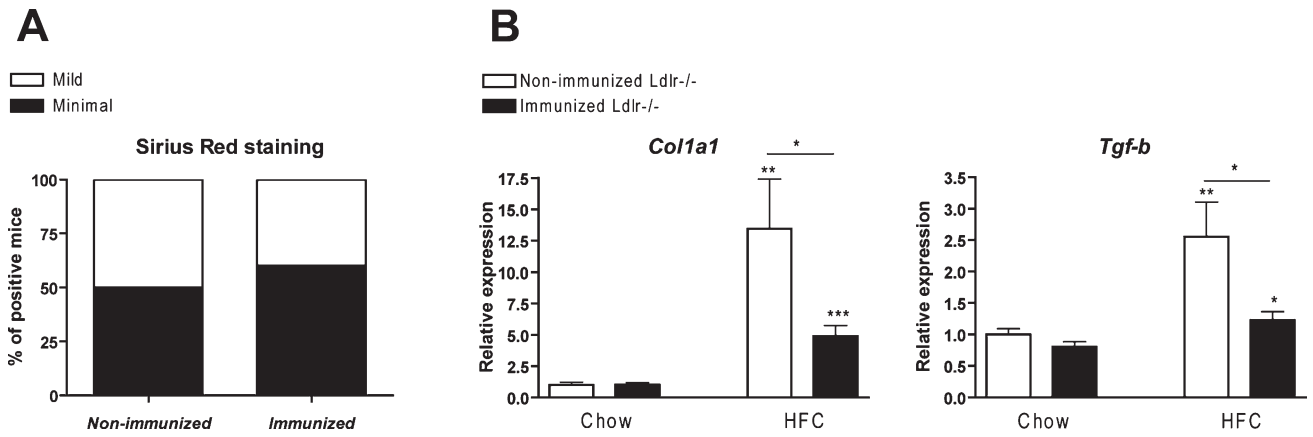

Fig. 5. Parameters of hepatic fibrosis. (A) Quantification of sirius red (collagen) after 3 weeks of an HFC diet. Livers were quantified as minimally, mildly, or moderately positive for collagen around and in between the blood vessels of the liver. (B) Gene expression analysis of the fibrosis markers, collagen (*Col1A1*) and *Tgf-β*. Asterisks indicate significant difference from nonimmunized mice on a chow diet. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

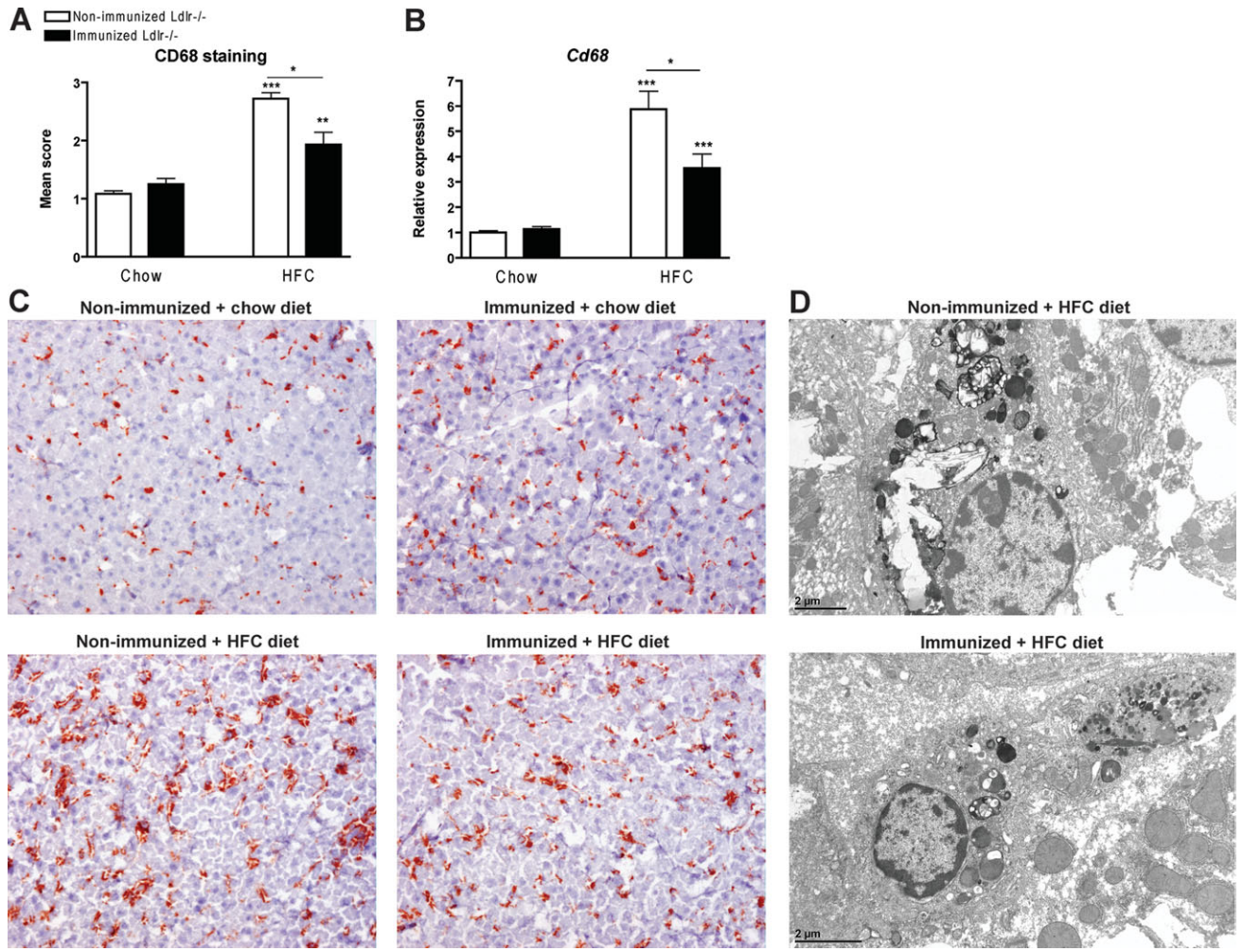

Fig. 6. Foamy KCs. (A) Liver sections were stained for CD68 (KCs) and scored for the level of foamy appearance: 1 (mild foamy appearance) to 3 (severe foamy appearance). Mean scores were calculated from six microscopic views. (B) Gene expression analysis of the KC activation marker CD68. (C) Representative images of liver sections stained for CD68 for $Ldlr^{-/-}$ mice on a chow diet without and with immunization and for $Ldlr^{-/-}$ mice on an HFC diet without and with immunization, respectively (magnification ×200). (D) Electron microscopy of foamy KCs. Acid phosphatase staining indicating the lysosomes of the KCs in nonimmunized and immunized $Ldlr^{-/-}$ mice on an HFC diet. Asterisks indicate significant difference from nonimmunized mice on a chow diet. $*P < 0.05$; $***P < 0.001$.

oxLDL. Therefore, it has been suggested that the elevated levels of lipid peroxidation might make an important contribution to the pathogenesis of NASH.[29,30] In the literature, it is demonstrated that the presence of immune responses toward lipid peroxidation products can be a predictor of progression of NAFLD.[31] In addition, it was demonstrated that oxidized phosphatidylcholines were found predominantly in steatotic hepatocytes and macrophages/KCs and were more abundant in NAFLD/NASH livers than in normal control livers.[32] Moreover, we have shown that NASH patients display increased hepatic myeloperoxidase activity, which is also associated with lipid peroxidation.[33] The role of oxidative stress as a key factor contributing to hepatic injury in patients with NASH[34,35] has been underlined by a study with vitamin E therapy of nondiabetic NASH patients.[36]

Because fibrosis is one of the later consequences of NASH, we investigated the effect of immunization with heat-inactivated pneumococci on hepatic fibrosis. Gene expression of fibrosis-related genes was decreased, yet not confirmed by sirius red staining. This is probably due to the short period of 3 weeks on an HFC diet, because *Ldlr*$^{-/-}$ mice only develop fibrosis after 3 months on a mild atherogenic diet.[4] However, we report for the first time that uptake of oxLDL is associated with fibrogenesis in vivo. In line with these observations, a study by Kang et al. demonstrated that oxLDL can activate hepatic stellate cells in vitro.[37] These findings indicate a crucial role for oxLDL in the fibrogenic process.

As expected, immunized mice on an HFC diet showed decreased foamy KCs compared with nonimmunized mice. This reduction in size is probably due to decreased plasma cholesterol levels, as the size of the foamy KCs is not always correlated with the inflammatory state of the liver.[4,16] Overloading of macrophages with oxLDL was shown to lead to the formation of cholesterol monohydrate crystals.[38] In line with these findings, we showed that after immunization with heat-inactivated pneumococci, KCs were less foamy, had less lysosomal cholesterol accumulation and therefore also less cholesterol crystals. These data indicate that the increased cholesterol accumulation inside KCs, together with the crystallization, is linked to hepatic inflammation.

To date, no therapy for NASH is available. Our novel data in mice suggest that future research should focus on oxLDL as a trigger for NASH. Therefore, the potential of PC-based vaccination strategies to be used as a novel tool for the prevention and therapy of NASH should be tested in the future.

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References

1. McCullough AJ. The clinical features, diagnosis and natural history of nonalcoholic fatty liver disease. Clin Liver Dis 2004;8:521-533.
2. Clark JM, Diehl AM. Defining nonalcoholic fatty liver disease: implications for epidemiologic studies. Gastroenterology 2003;124:248-250.
3. Parekh S, Anania FA. Abnormal lipid and glucose metabolism in obesity: implications for nonalcoholic fatty liver disease. Gastroenterology 2007;132:2191-2207.
4. Bieghs V, Wouters K, van Gorp PJ, Gijbels MJ, de Winther MP, Binder CJ, et al. Role of scavenger receptor A and CD36 in diet-induced nonalcoholic steatohepatitis in hyperlipidemic mice. Gastroenterology 2010;138:2477-2486.
5. Yamada Y, Doi T, Hamakubo T, Kodama T. Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nervous system. Cell Mol Life Sci 1998;54:628-640.
6. Karvonen J, Paivansalo M, Kesaniemi YA, Horkko S. Immunoglobulin M type of autoantibodies to oxidized low-density lipoprotein has an inverse relation to carotid artery atherosclerosis. Circulation 2003;108:2107-2112.
7. Horkko S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, et al. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. J Clin Invest 1999;103:117-128.
8. Tsimikas S, Brilakis ES, Lennon RJ, Miller ER, Witztum JL, McConnell JP, et al. Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. J Lipid Res 2007;48:425-433.
9. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, et al. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. J Clin Invest 2009;119:1335-1349.
10. Lutz HU, Binder CJ, Kaveri S. Naturally occurring auto-antibodies in homeostasis and disease. Trends Immunol 2009;30:43-51.
11. Binder CJ, Silverman GJ. Natural antibodies and the autoimmunity of atherosclerosis. Springer Semin Immunopathol 2005;26:385-404.
12. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. Nat Rev Immunol 2011;11:34-46.
13. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J Clin Invest 2000;105:1731-1740.
14. Palinski W, Horkko S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK, et al. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. J Clin Invest 1996;98:800-814.
15. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nat Med 2003;9:736-743.
16. Wouters K, van Gorp PJ, Bieghs V, Gijbels MJ, Duimel H, Lutjohann D, et al. Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. HEPATOLOGY 2008;48:474-486.
17. Briles DE, Forman C, Hudak S, Claflin JL. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. J Exp Med 1982;156:1177-1185.

18. Itabe H, Suzuki K, Tsukamoto Y, Komatsu R, Ueda M, Mori M, et al. Lysosomal accumulation of oxidized phosphatidylcholine-apolipoprotein B complex in macrophages: intracellular fate of oxidized low density lipoprotein. Biochim Biophys Acta 2000;1487:233-245.
19. Ehara S, Ueda M, Naruko T, Haze K, Itoh A, Otsuka M, et al. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation 2001; 103:1955-1960.
20. Stewart CR, Stuart LM, Wilkinson K, van Gils JM, Deng J, Halle A, et al. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nat Immunol 2010;11: 155-161.
21. Platt N, Suzuki H, Kurihara Y, Kodama T, Gordon S. Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. Proc Natl Acad Sci U S A 1996;93:12456-12460.
22. Febbraio M, Hajjar DP, Silverstein RL. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. J Clin Invest 2001;108:785-791.
23. Cotena A, Gordon S, Platt N. The class A macrophage scavenger receptor attenuates CXC chemokine production and the early infiltration of neutrophils in sterile peritonitis. J Immunol 2004;173:6427-6432.
24. Caligiuri G, Khallou-Laschet J, Vandaele M, Gaston AT, Delignat S, Mandet C, et al. Phosphorylcholine-targeting immunization reduces atherosclerosis. J Am Coll Cardiol 2007;50:540-546.
25. Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, et al. Innate and acquired immunity in atherogenesis. Nat Med 2002; 8:1218-1226.
26. Chang MK, Bergmark C, Laurila A, Horkko S, Han KH, Friedman P, et al. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. Proc Natl Acad Sci U S A 1999;96:6353-6358.
27. Kayo S, Ohsawa M, Ehara S, Naruko T, Ikura Y, Hai E, et al. Oxidized low-density lipoprotein levels circulating in plasma and deposited in the tissues: comparison between Helicobacter pylori-associated gastritis and acute myocardial infarction. Am Heart J 2004;148:818-825.
28. Hartvigsen K, Chou MY, Hansen LF, Shaw PX, Tsimikas S, Binder CJ, et al. The role of innate immunity in atherogenesis. J Lipid Res 2009; 50(Suppl.):S388-S393.
29. Chalasani N, Deeg MA, Crabb DW. Systemic levels of lipid peroxidation and its metabolic and dietary correlates in patients with nonalcoholic steatohepatitis. Am J Gastroenterol 2004;99:1497-1502.
30. James O, Day C. Non-alcoholic steatohepatitis: another disease of affluence. Lancet 1999;353:1634-1636.
31. Albano E, Mottaran E, Vidali M, Reale E, Saksena S, Occhino G, et al. Immune response towards lipid peroxidation products as a predictor of progression of non-alcoholic fatty liver disease to advanced fibrosis. Gut 2005;54:987-993.
32. Ikura Y, Ohsawa M, Suekane T, Fukushima H, Itabe H, Jomura H, et al. Localization of oxidized phosphatidylcholine in nonalcoholic fatty liver disease: impact on disease progression. HEPATOLOGY 2006;43:506-514.
33. Rensen SS, Slaats Y, Nijhuis J, Jans A, Bieghs V, Driessen A, et al. Increased hepatic myeloperoxidase activity in obese subjects with nonalcoholic steatohepatitis. Am J Pathol 2009;175:1473-1482.
34. Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, et al. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology 2001;120:1183-1192.
35. McClain CJ, Mokshagundam SP, Barve SS, Song Z, Hill DB, Chen T, et al. Mechanisms of non-alcoholic steatohepatitis. Alcohol 2004;34:67-79.
36. Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. N Engl J Med;362:1675-1685.
37. Kang Q, Chen A. Curcumin eliminates oxidized LDL roles in activating hepatic stellate cells by suppressing gene expression of lectin-like oxidized LDL receptor-1. Lab Invest 2009;89:1275-1290.
38. Tangirala RK, Jerome WG, Jones NL, Small DM, Johnson WJ, Glick JM, et al. Formation of cholesterol monohydrate crystals in macrophage-derived foam cells. J Lipid Res 1994;35:93-104.